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Antibody Against the Epstein-Barr Virus BHRF1 Protein, a Homologue of Bcl-2, in Patients With Nasopharyngeal Carcinoma

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The Epstein-Barr virus (EBV) open reading frame BHRF1, a homologue of the oncogene bcl-2, was cloned from a patient with nasopharyngeal carcinoma (NPC) and overexpressed in *Escherichia coli.* The resulting recombinant BHRF1 fusion protein, with an apparent molecular weight of 35 KD, was used as antigen in an immunoblotting assay for IgG antibody in human sera. Anti-BHRF1 antibody was detected in 57 (61.3%) of 93 patients with NPC, 5 (5.7%) of 87 patients with nonmalignant diseases of the nasopharynx, and in 1 (1.3%) of 78 healthy blood donors. The positivity rate in these nonmalignant patients was 4.4 times that of the normal controls. Negative results were observed in four patients with infectious mononucleosis and patients with other cancers, including 4 with esophageal cancer, 11 with lung cancer, 10 with lymphoma, 13 with gastric carcinoma, 10 with cervical carcinoma, and 10 with other head and neck cancers. Antibody neutralizing EBV DNase and IgA antibody to viral capsid antigen (VCA) were assayed in parallel. The results showed that 7.5% of the NPC patients were negative for anti-DNase and anti-VCA antibodies and EBV infection could be detected by the anti-BHRF1 antibody alone. The demonstration of anti-BHRF1 antibody in most NPC sera strongly supports the hypothesis that the EBV BHRF1 protein is expressed in most NPC patients and its specific antibody can be a useful marker for the diagnosis of NPC. ***J. Med. Virol. 56:179–185, 1998.*** © 1998 Wiley-Liss, Inc.

KEY WORDS: nasopharyngeal carcinoma; anti-EBV BHRF1 antibody

INTRODUCTION

Epstein-Barr virus (EBV) is an important human virus from both the clinical and virological aspects. Primary infection in young adults in the Western world can cause infectious mononucleosis (IM) [Henle et al., 1968] and infection in childhood sometimes results in the occurrence of hemophagocytic syndrome, which may be lethal [Chen et al., 1991]. EBV has also been reported to be associated with several human cancers, including Burkitt's lymphoma, which is an epidemic cancer of African children [de The, 1979], B-cell lymphoma in immunocompromised patients [Shapiro et al., 1988], T-cell lymphoma [Su and Hsieh, 1988], Hodgkin's disease [Pallesen et al., 1991], and gastric cancer [Oda et al., 1993]. Most significantly, infection of EBV is closely associated with nasopharyngeal carcinoma (NPC) [Old et al., 1966], which is a prevalent malignancy in the southern Chinese population [Shanmugaratnam, 1982].

Transcripts of several EBV lytic genes, such as BRLF2, BMLF1, and BLLF1, were detected in some NPC biopsies [Martel-Renoir et al., 1995]. Expression of the EBV BZLF1 gene, which encodes an immediate early transactivator, was detected rarely in NPC biopsies [Niedobitek et al., 1992]. However, serological data revealed that 87% of NPC sera contained antibodies to the BZLF1 protein [Joab et al., 1991]. Other serological investigations showed that high-titer IgA antibodies to EBV early antigen (EA) and viral capsid antigen (VCA) were present in sera of most NPC patients [Henle and Henle, 1979; Pearson et al., 1983]. These observations suggest prior or ongoing EBV infection in NPC patients.

BHRF1, another lytic gene, encodes a 17 KD component of the EBV-restricted early antigen complex (EA-

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R) [Pearson et al., 1987] and has been postulated to be closely associated with the development of NPC, based on the following observations: alignment of amino acid sequences showed homology between the BHRF1 and bcl-2, a cellular protooncogene [Cleary et al., 1986; Hickish et al., 1994]; Bcl-2 protein is able to suppress cell death induced by apoptosis [Vaux et al., 1988; Garcia et al., 1992; Allsopp et al., 1993]. The BHRF1 protein was shown to be a functional analogue of the Bcl-2 protein in its ability to prevent programmed death of human B-cells [Henderson et al., 1993; Tarodi et al., 1994; Dawson et al., 1995]. Furthermore, the cellular distributions of the BHRF1 and Bcl-2 proteins are similar, both localizing at the periphery of mitochondria [Hickish et al., 1994]. These findings indicate that the gene products of BHRF1 and bcl-2 are functionally and mechanistically similar to each other.

The role of BHRF1 in NPC carcinogenesis has been suggested to be through prevention of apoptosis of EBV-infected cells during the early stages of cancer development [Horner et al., 1995]. If this hypothesis is true, there may be increased expression of the BHRF1 protein in NPC patients. Consequently, anti-BHRF1 antibody should be present in NPC patients and might be a good marker for NPC diagnosis. To test this hypothesis, we cloned the BHRF1 gene from an NPC patient and expressed it in *Escherichia coli.* The recombinant BHRF1 protein was then used as antigen to detect specific antibody in human sera by Western blotting. It was found that antibody against the BHRF1 protein was detected specifically in NPC patients.

MATERIALS AND METHODS

Sera

Sera from patients with various malignancies, including 93 sera from patients with NPC, 4 esophageal cancer, 11 lung cancer, 10 lymphoma, 13 gastric carcinoma, 10 cervical carcinoma, and 10 other head and neck cancers, were collected from two large hospitals in Taipei, Taiwan: the National Taiwan University Hospital and the MacKay Memorial Hospital. Patients with IM are rare in Taiwan and only four sera were collected. In addition, sera from 87 patients without malignancies, and whose nasopharyngeal biopsies showed negative pathology for NPC, were used as non-NPC controls. These patients underwent nasopharyngeal biopsy because they had symptoms mimicking NPC, such as blood-tinged rhinorrhea, otitis media with effusion, cervical lymphadenopathy, or nasopharyngeal lesions. Sera from 78 healthy blood donors were used as normal controls and a serum from a young child without EBV infection was taken as a negative control. All the serum samples were inactivated at 56°C for 30 min and stored at –20°C prior to testing for anti-EBV antibodies.

Amplification of BHRF1 Gene From NPC Biopsy and Construction of Recombinant Plasmid

A nasopharyngeal biopsy was obtained from a patient who was subsequently confirmed with NPC by histological analysis. High-molecular-weight DNA was extracted from the tissue using TRIZOL reagent (GIBCO-BRL, Grand Island, NY) according to the manufacturer's instructions. Endogenous EBV DNA was amplified using the polymerase chain reaction (PCR) with primers specific for the BHRF1 open reading frame. The primer sequences were 5′-GGAATTCGTCGACCAGATCTTGTTGAGCAAG-3′ and 5′-TAAAGTGCTCGAGAAAATGT-3′, flanking the 5′ end and the 3′ end of the gene, respectively. Restriction enzyme sites for endonucleases *Eco*RI (GAATTC) and *Xho*I (CTCGAG) were included in the primers. The PCR was carried out with 40 cycles of incubation at 94°C for 1 min, 44°C for 1 min, and 72°C for 1.5 min. A 634 bp PCR product was produced and digested with *Eco*RI and *Xho*I. The resulting 624 bp *Eco*RI-*Xho*I DNA fragment was purified by agarose gel electrophoresis and isolated from the gel by using a Jetsorb kit (GENOMED, Research Triangle Park, NC). The fragment was inserted between the *Eco*RI and *Xho*I sites of an expression vector, pET-32a (Novagen, Madison, WI), and the recombinant plasmid was designated as pYYS-2 (Fig. 1).

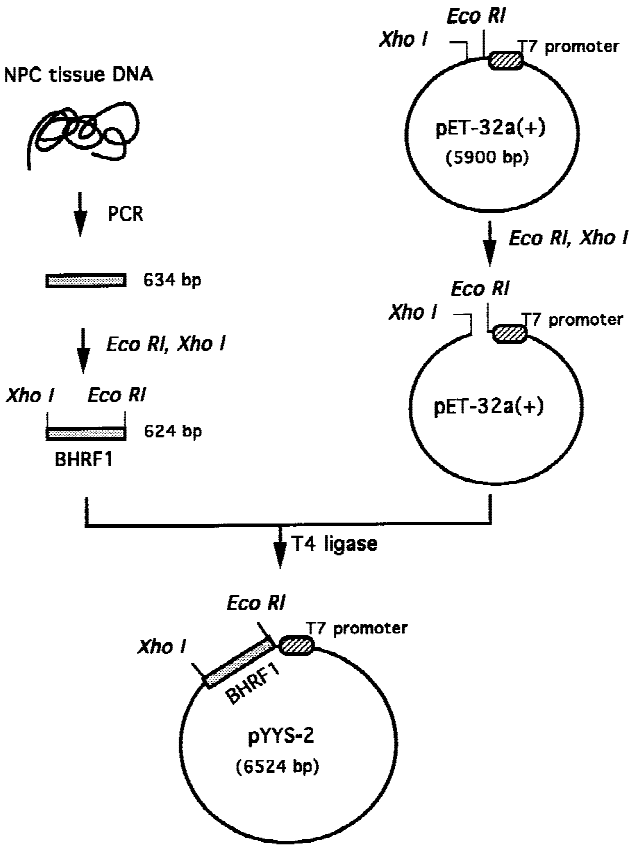


Fig. 1. Construction of the BHRF1 expression vector. The BHRF1 DNA fragment from an EBV-positive NPC tissue was amplified by PCR and then digested with *Eco*RI and *Xho*I. The *Eco*RI-*Xho*I fragment was inserted in the vector pET-32a, giving the recombinant plasmid, pYYS-2.

Expression of Recombinant BHRF1 Protein

A T_7 RNA polymerase expression system [Studier and Moffat, 1986] was used to express the recombinant

BHRF1 protein in bacteria. The pYYS-2 plasmid was introduced into *E. coli* strain BL21(DE3) by transformation. A single transformant was grown in ampicillin (200 μg/ml)-containing medium until the culture reached an absorbance of 0.4–0.6 at 600 nm. Expression of the recombinant BHRF1 protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM to initiate synthesis of the T_7 RNA polymerase, which in turn transcribed the BHRF1 gene. Three hours after induction the bacteria were harvested and tested for production of the expected recombinant protein.

Electrophoresis and Western Blot Analysis

To analyze the expressed protein, total cell protein samples were prepared by boiling the bacteria in 2% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, 50 mM Tris-HCl (pH 6.8), 10% glycerol, and 0.3% bromophenol blue. The protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and were then stained with Coomassie brilliant blue or transferred electrophoretically to Hybond-C super membrane (Amersham, Buckinghamshire, UK). For Western blot analysis, the blotted membranes were reacted with anti-BHRF1 monoclonal antibody, mAb 5B11 (ABi, Columbia, MD), or human sera at 1:200 dilution according to the procedures described by Towbin et al. [1979]. Horseradish peroxidase-conjugated antibody (CAPPEL, Organon Teknika, NC) was used as the secondary antibody to react with the primary antibodies on blots. The blots were then developed using an ECL Western blotting detection system (Amersham, Buckinghamshire, UK), in which a peroxidase-catalyzed oxidation of luminol and subsequently enhanced chemiluminescence are elicited. The developed blots were then exposed to an X-ray film and the results were quantified by scanning the film using the program FREEMAX version 3.0 (Media Cybernetics, MD). The values obtained were classified into four categories as follows: readings less than 0.5 were designated as (–); equal to 0.5 or between 0.5 and 1.0 as (+/–); equal to 1.0 or between 1.0 and 2.0 as (+); equal to or more than 2.0 as (++). Positive reactions were considered for sera with reactivity of (+) or (++).

Indirect Immunofluorescence Assay

Smears of P3HR1 cells (an EBV positive cell line) were prepared to detect human IgA antibody against the EBV VCA. Briefly, P3HR1 cells were seeded at 10^6 cells per ml in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum. To induce EBV gene expression, 5-iodo-2′-deoxyuridine (IdUrd) was added to a final concentration of 60 μg/ml. After three days of incubation, IdUrd was removed by replacing with fresh medium and the cells were cultured for one more day. The cells were then harvested, washed with phosphate buffered saline (PBS), and fixed on slides by acetone [Henle and Henle, 1966]. The smears were incubated with appropriately diluted human sera at 37°C for 1 hr in a humidified atmosphere. After washing with PBS, fluorescein isothiocyanate (FITC)-conjugated goat antihuman IgA was used as the secondary antibody. Fluorescein-tagged cells were visualized by excitation at 450–490 nm using a UV photomicroscope. A titer less than 1:10 was considered to be negative for anti-VCA IgA antibody.

DNase Neutralization Test

IdUrd-induced P3HR1 cells were resuspended in extraction buffer (50 mM Tris-HCl, pH 7.5, 0.3 M KCl, 5 mM β-mercaptoethanol, 0.7 mM phenylmethylsulfonyl fluoride, and 20% glycerol) and frozen and thawed three times. The lysate was spun and the supernatant was collected as the enzyme source for DNase. One unit of DNase activity was defined as the amount of enzyme that converted 1 μg of double-stranded DNA to acid-soluble material in 10 min at 37°C. Levels of DNase-neutralizing antibody in human sera were determined by incubating the sera with the enzyme and then assaying residual DNase activity as described by Chen et al. [1985]. The level of anti-DNase antibody was expressed in terms of the units of DNase activity neutralized by 1 ml serum. Neutralization activity less than two units was considered to be negative.

RESULTS

Cloning and Expression of BHRF1 Gene in *E. coli*

The recombinant plasmid pYYS-2 contains an insert contiguous with coordinates 54,358 to 54,960 of the EBV (B95-8 strain) genome. This fragment covers the full length of the BHRF1 open reading frame (54,376–54,948). Authenticity of the 5′ end sequence of the insert was confirmed by DNA sequencing (data not shown). After introduction of the pYYS-2, *E. coli* BL21(DE3) was cultured in the presence of IPTG to induce synthesis of the BHRF1 protein. A protein of 35 KD, which resulted from the fusion of an 18 KD tag and the 17 KD EA-R, was detected, while only the 18 KD tag protein was produced from the vector-transformed cells (Fig. 2A). To confirm the expression of BHRF1, mAb 5B11 was reacted with the 35 KD protein by Western blotting. Figure 2B shows that the recombinant product could be recognized by the monoclonal antibody and so possessed the authentic antigenicity of the BHRF1 protein.

Western Blot Analyses of Anti-BHRF1 Antibody in Human Sera

To determine the seroprevalence of anti-BHRF1 antibody, sera from various patients and controls were examined by Western blotting using the BHRF1 fusion protein as antigen. mAb 5B11 and an EBV negative human serum were used as positive and negative controls on each blot. Representative results are shown in Figure 3. The sera under test were reacted with proteins from BHRF1-transformed *E. coli* (Fig. 3A–D) and vector-transformed *E. coli* (Fig. 3E,F). To compare the sensitivity for different classes of immunoglobulins, IgA (Fig. 3A,B) and IgG (Fig. 3C,D) were detected separately. The results show that reactivity of IgG was

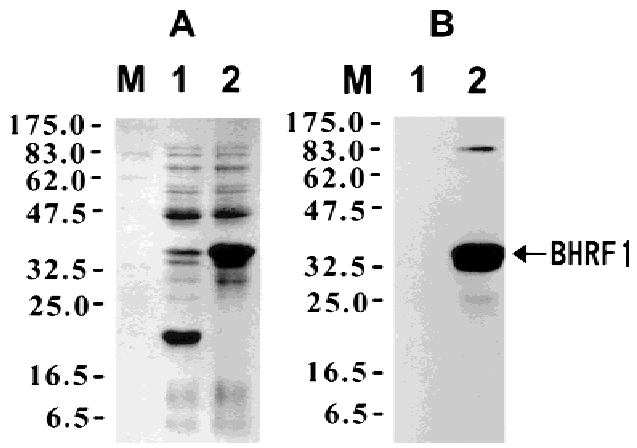


Fig. 2. Expression of recombinant BHRF1 protein in *E. coli.* Plasmid pYYS-2 was introduced into *E. coli* BL21(DE3) and induced by IPTG for 3 hr. Protein extracts of the cell lysates were fractionated by SDS-PAGE (**A**) and probed with monoclonal antibody 5B11 against the BHRF1 protein by Western blotting (**B**). Lane 1 was loaded with *E. coli* transformed by the vector pET-32a as a negative control and lane 2 was loaded with the pYYS-2-transformed cells. M denotes the size markers with molecular weights indicated in KD.

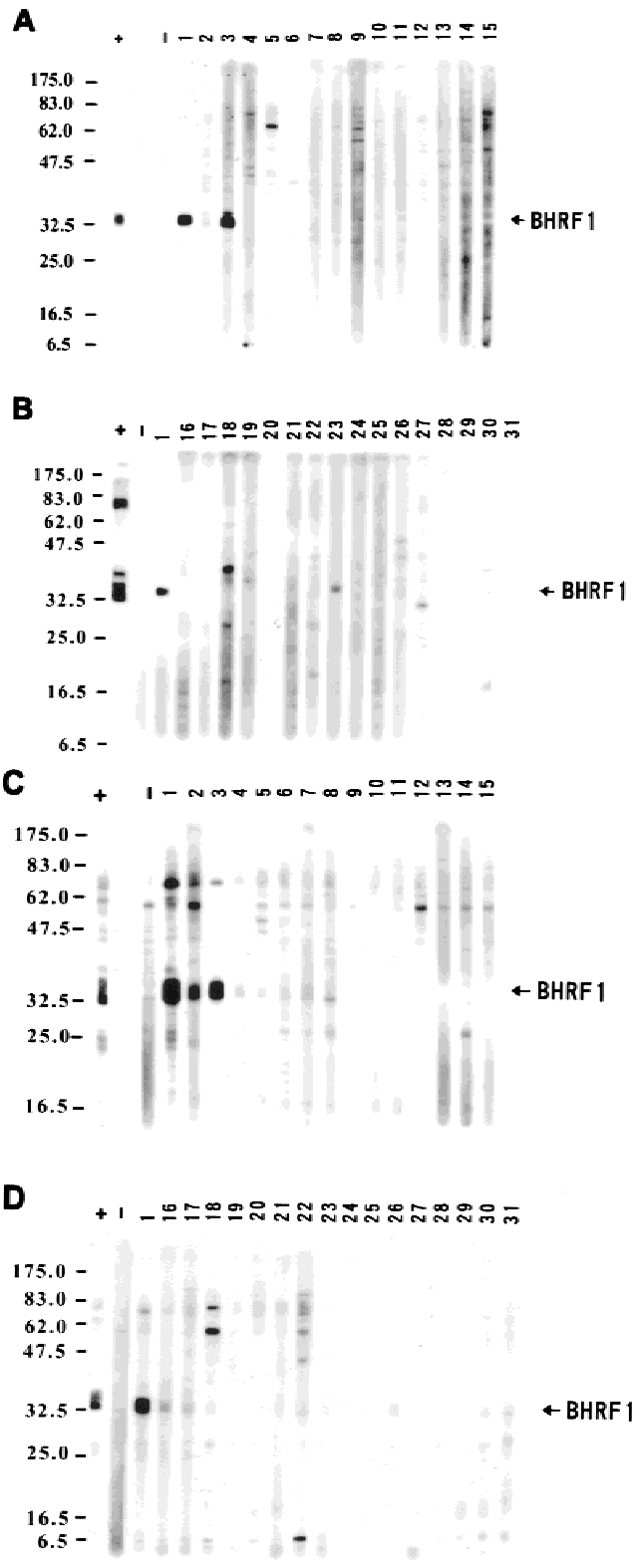
stronger than that of IgA in that more intense signals were obtained for IgG. As for the specificity for NPC patients, both IgA and IgG appeared to be good. Based on the IgG data shown in Figure 3C and 3D, positive reactions were observed for NPC sera (lanes 1–3) and negative results were obtained for the rest of the sera (lanes 4–31), including sera from patients with other cancers, nonmalignant nasopharyngeal diseases, IM, and healthy donors. To exclude the possibility that the 35 KD reacting band was derived from a comigrating *E. coli* protein, the same sera were reacted with proteins from the vector-transformed *E. coli* and IgG was monitored (Fig. 3E,F). Neither a 35 KD band comigrating with the recombinant BHRF1 protein nor a 17 KD band of the tag protein could be detected on the gel. The results clearly proved that the positive reactions shown in Figure 3C and 3D were specific antibody responses to the BHRF1 protein rather than to an *E. coli* protein or the tag protein. The results of the Western blot analyses are summarized in Table I. Four categories of anti-BHRF1 IgG antibody reactivity were defined as described in Materials and Methods. If all the indeterminate results (+/–) were regarded as negative, 61.3% of the NPC patients were positive for the IgG antibody. In contrast, 5.7% of the nonmalignant patients with symptoms mimicking NPC and 1.3% of the normals were positive. The positivity rate of the NPC group was significantly higher than that of the nonmalignant group ($P = 0$) and that of the healthy group ($P = 0$) when Fisher's exact test was used for the statistical analysis. The antibody was absent from the other cancer patients tested. Furthermore, the four EBV-related IM patients were also negative for the antibody. These results demonstrate that anti-BHRF1 antibody is present in NPC patients specifically.

Antibodies Against Other Lytic Proteins of EBV

Many lytic proteins, such as those required for nucleic acid metabolism and virion assembly, are synthesized in the process of EBV replication. Among them, early protein DNase and late protein VCA have been employed extensively in the diagnosis and early detection of NPC. Antibodies against these two proteins have been shown to be elevated in most NPC patients. To compare the immune response to various EBV lytic proteins, antibody neutralizing the DNase activity and IgA antibody to the VCA were also determined for the NPC patients, the normal controls, and those who showed symptoms mimicking NPC but had negative pathology. The positivity rates are shown in Table II. Both anti-VCA IgA and DNase-neutralizing antibodies are more prevalent than anti-BHRF1 antibody in patients with NPC. Nearly half of the NPC patients (46.2%) were positive for all three antibodies, and only 4.3% were negative for all the tests. The remaining NPC patients were positive for at least one antibody, indicating that most of the tested NPC patients were infected with EBV and EBV elicited varying immune responses in different individuals. In the control groups, 71.3% of the patients with nonmalignant diseases around their nasopharynx and 76.9% of the healthy donors were negative for all three antibodies. Among the healthy donors, no one was positive for all three tests and only 4.6% of the nonmalignant patients were positive for any antibody tested.

DISCUSSION

Studies on EBV recombinants indicated that BHRF1 is not required for virus replication or virus-induced B-cell transformation [Marchini et al., 1991; Lee and Yates, 1992]. However, experiments in vitro demonstrated that BHRF1 was able to perturb normal epithelial cell differentiation by preventing or delaying the onset of apoptosis [Dawson et al., 1995]. The pathogenic role of BHRF1 in the carcinogenesis of nasopharyngeal epithelium in vivo is not well understood. If BHRF1 was expressed in infected epithelia with an equivalent activity to that displayed in vitro, it might contribute to creating a cellular environment in which expression of EBV latent genes could be maintained and where other genetic events could occur. Hence, expression of BHRF1 may be necessary for the establishment of NPC. Evidence of BHRF1 expression was sought in NPC patients and controls to test this hypothesis. Expression of BHRF1 can be proved directly, by detecting its transcripts in tumor tissues, or indirectly, by detecting anti-BHRF1 antibody in NPC sera. This report is the first to demonstrate that anti-BHRF1 antibody was present in sera of most NPC patients. In a separate experiment, we were able to detect the presence of BHRF1 transcripts in a large proportion of NPC biopsies (data not shown). Together with our previous understanding of the antiapoptosis activity of BHRF1, our work strongly supports the hypothesis that BHRF1 may play some role in the development of NPC.



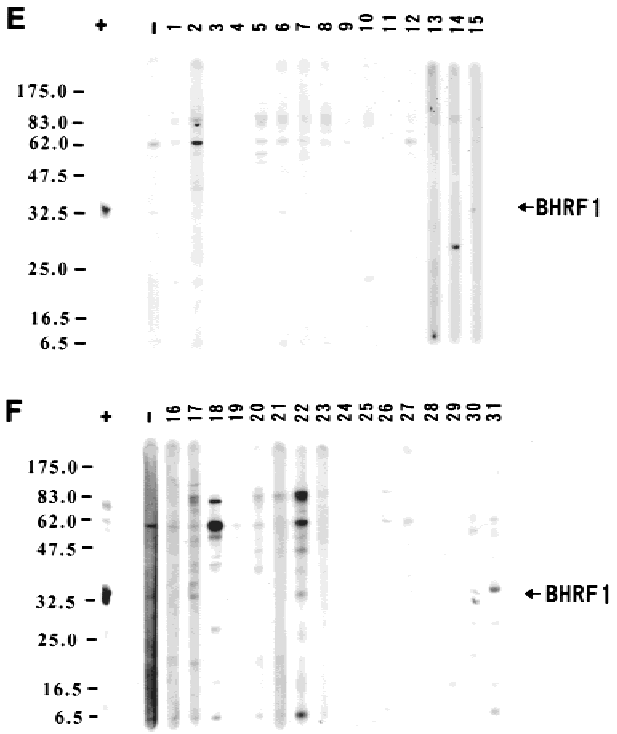


Fig. 3. Western blot analysis of anti-BHRF1 antibody in human sera. Cell extracts from BHRF1-expressing cells (**A–D**) and vector-transformed cells (**E** and **F**) were blotted onto membranes and reacted with various sera. Lanes 1–3 are sera from NPC patients, 4–6 from patients with symptoms mimicking NPC but negative pathology, 7–9 from healthy donors, 10–12 from cervical carcinoma, 13–15 from gastric carcinoma, 16–18 from other head and neck cancer, 19–21 from lymphoma, 22–24 from esophageal cancer, 25–27 from lung cancer, and 28–31 from patients with IM. Horseradish peroxidase-conjugated antihuman IgA (A and B) and IgG (C–F) antibodies were used as the secondary antibodies, respectively. (+), mAb 5B11 against the BHRF1 protein was used as a positive control, for which horseradish peroxidase-conjugated antimouse IgG antibody was the secondary antibody. (–), an EBV-uninfected child serum was used as a negative control.

Seroepidemiological studies on anti-BHRF1 antibody are rare. Pothen et al. [1993] synthesized three peptides derived from the BHRF1 protein sequence and reacted them with human sera using an ELISA format. Patients with aggressive lymphomas showed a high positivity rate (29–82%, depending on the peptide applied). Surprisingly, NPC patients were less reactive, with positivity rates ranging from 13% to 23%. In our work, the full-length protein was used as antigen and the highest rate was obtained in NPC patients. The rate for the lymphoma patients tested was 0%. However, only 10 lymphoma patients were examined and just one gave an indeterminate reaction. The discrepancy between the two study groups may be due to different detection methods applied; sensitivity and specificity could vary between ELISA and Western blotting. Another possibility is that different forms of antigen were used in these two assays. The entire BHRF1 protein molecule was used in the Western blot analysis and only few polypeptides were applied in the ELISA. Newkirk et al. [1996] reported that a subgroup of rheumatic disease patients, primarily those with Sjogren's syndrome, were more reactive than normal controls in their immune response to a synthetic peptide of BHRF1. Therefore, the BHRF1 protein has been suggested to contribute to the lymphoproliferative nature of rheumatic diseases. Since synthetic peptides were also used in that study, the correlation between EBV BHRF1 protein and rheumatic diseases may better be confirmed with the whole molecule.

Based on the data from NPC patients and healthy donors, Western blot analysis can reach a sensitivity of 61.3% and a specificity of 98.7%. There was a small

TABLE I. Anti-EBV BHRF1 Antibody in Human Sera

Sera	Number tested	Relative reactivity ++	+	+/–	–	Positive[a] number	Positivity rate
NPC	93	31	26	17	19	57	61.3%
Non-NPC[b]	87	1	4	4	78	5	5.7%
Healthy donors	78	0	1	5	72	1	1.3%
Infectious mononuleosis	4	0	0	0	4	0	0.0%
Other cancers:	58	0	0	2	56	0	0.0%
Esophageal cancer	4	0	0	0	4	0	0.0%
Lung cancer	11	0	0	0	11	0	0.0%
Lymphoma	10	0	0	1	9	0	0.0%
Gastric carcinoma	13	0	0	1	12	0	0.0%
Cervical carcinoma	10	0	0	0	10	0	0.0%
Other head and neck cancer	10	0	0	0	10	0	0.0%

[a]Patients with ++ or + reactivity are considered to be positive.
[b]Patients with symptoms mimicking NPC but negative pathology.

TABLE II. Comparison of Antibodies Against BHRF1 and Other EBV Lytic Proteins

Presence of Antibody to BHRF1[a]	DNase[b]	VCA[c]	NPC patients (n = 93)	Non-NPC patients[d] (n = 87)	Healthy donors (n = 78)
+			56 (61.3%)	5 (5.7%)	1 (1.3%)
	+		79 (84.0%)	23 (26.4%)	14 (17.7%)
		+	65 (69.1%)	9 (10.3%)	6 (7.6%)
+	+	+	43 (46.2%)	4 (4.6%)	0 (0.0%)
+	+	–	7 (7.5%)	1 (1.1%)	0 (0.0%)
+	–	+	0 (0.0%)	0 (0.0%)	0 (0.0%)
–	+	+	17 (18.3%)	3 (3.4%)	3 (3.8%)
+	–	–	7 (7.5%)	0 (0.0%)	1 (1.3%)
–	+	–	11 (11.8%)	15 (17.2%)	11 (14.1%)
–	–	+	4 (4.3%)	2 (2.3%)	3 (3.8%)
–	–	–	4 (4.3%)	62 (71.3%)	60 (76.9%)

[a]IgG antibody determined by Western blotting.
[b]Total antibody determined by DNase activity neutralization test.
[c]IgA antibody determined by indirect immunofluorescence assay.
[d]Patients with symptoms mimicking NPC but negative pathology.

portion of the NPC patients who could only be detected by the anti-BHRF1 antibody but not by conventionally used anti-VCA IgA or DNase-neutralizing antibodies. It appears that the anti-BHRF1 antibody will be a useful complementary marker for NPC diagnosis. Patients with symptoms similar to NPC but with negative pathology showed stronger antibody reactions to the BHRF1 protein than did the normal controls. It is noteworthy that the positivity rate of the nonmalignant patients is 4.4 times that of the normals. Reactivation of EBV may have occurred in some of these patients. Assessment of whether these individuals are at high risk of developing NPC requires long-term follow-up. For the other cancer patients, only one lymphoma and one gastric carcinoma patient showed very weak reactions. These two cancers were also reported to have some association with EBV infection [Shapiro et al., 1988; Su and Hsieh, 1988; Oda et al., 1993]. However, the intensities of their responses were not as strong as those displayed by the NPC patients and were classified as negative by our stringent criteria. The four IM patients were all negative for the anti-BHRF1 antibody, although they were all positive for IgM and IgG antibodies to VCA (data not shown). It is interesting that antibody to the BHRF1 early protein cannot be found in IM patients during acute EBV infection. This finding further supports that the anti-BHRF1 antibody provides specificity for NPC diagnosis.

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